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Reconstructing Prions: Fibril Assembly from Simple Yeast to Complex Mammals

Christina Sigurdson Magdalini Polymenidou Adriano Aguzzi

Institute of Neuropathology, University Hospital of Zürich, Zürich, Switzerland

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Abstract
With the epizootics of bovine spongiform encephalopathy (BSE) in North American cattle, BSE infections in goats, new forms of human Creutzfeldt-Jakob disease (CJD) and the spread of chronic wasting disease in North American deer and elk, one wonders whether we are gaining control over the transmissible spongiform encephalopathies (TSEs). Although many basic scientific questions in the prion field remain hotly debated and unresolved [1], including the function of the cellular prion protein (PrP), light has been shed on a diverse array of topics, and discussions at the latest TSE meeting ranged broadly from yeast prion fibril assembly to mammalian prion neurotoxicity to future TSE therapies. Prion diseases are protein misfolding disorders which cause degeneration of the central nervous system (CNS) and ultimately death. The unique and surprising feature is that prion diseases are infectious. Yeast prions are derived from proteins differing from the mammalian PrP but are also infectious, self propagating proteins which typically can convert into an aggregated, amyloidogenic form having high beta sheet content. The simple yeast organism has served as a valuable model for understanding aspects of prion biology, such as prion fibril assembly.

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Yeast Prions

How do monomeric prion molecules assemble into fibrils? The delicate balance of protein assembly and disassembly was addressed using an elegant model, the yeast prion Sup35 (S. Lindquist, Cambridge, Mass., USA). Since 1995, it is known that that the heat-shock protein HSP104 is required to catalyze Sup35 prion formation in yeast, and merely an excess or insufficient HSP104 abolishes Sup35 oligomers and fibrils [2]. Lindquist and colleagues could now elucidate the mechanism. HSP104 initiates the formation of the oligomeric intermediate, acting to nucleate the oligomers, but can also break up the amyloid fibers (together with ATP). Thus in high concen-

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Prof. Adriano Aguzzi
Institute of Neuropathology, University Hospital of Zürich
Schmelzbergstrasse 12
CH–8091 Zürich (Switzerland)
Tel. +41 44 255 2107, Fax +41 44 255 4402, E-Mail adriano@pathol.unizh.ch

trations of HSP104, the oligomerization and fibril formation are extinguished. So in the yeast system, HSP104 tightly controls the fibrillization and disassembly of the Sup35 yeast prion, either promoting fibril assembly or elimination – leading one to wonder whether perhaps similar regulatory mechanisms also exist in mammalian amyloid formation and elimination.

The process of prion aggregation may be determined by the amino acid content or the amino acid sequence. Wickner et al. [3] approached this question using another yeast prion, namely Ure2p. By shuffling the domains within Ure2p, several constructs were created, all of which led to prions and amyloid formation in vitro. Therefore, for Ure2p, prions were determined by amino acid content, not sequence. This amazing sequence independence of the prion domain suggests the formation of *parallel*, beta-pleated structures. Additionally, deletions in the C-terminal domain of Ure2p increased the frequency of fibril formation by 100×, supporting a model where an unknown factor tethers the C-terminal domain, maintaining a normal conformation.

In vertebrates, the situation is more complex, and in vitro conversion studies of the mammalian PrP have proven crucial. A key ingredient in prion disease development is undoubtedly the conversion of the normal cellular PrP into a pathogenic, highly protease-resistant isoform that aggregates and forms amyloid fibrils, pathologically in the brain. These aggregates lead to neurodegeneration and terminal disease. The quintessential puzzle has been the nature of the infectious agent: is it solely the PrP, or is PrP merely an indispensable component of the agent?

Prion Conversion in vitro: Scaffolds and Toxic Oligomers

How is the mammalian PrP converted to the misfolded prion, PrP^{Sc}? Can we amplify PrP^{Sc} in vitro to improve our diagnostic assay sensitivity? Surachai Supattapone (Hanover, N.H., USA) showed an in vitro prion conversion model in which affinity-purified preparations of PrP^{C} plus hamster or worm RNA led to impressive increases in the PrP^{Sc} content. While his original publication had suggested a sequence specificity to eukaryotic RNA [4], he now reported that synthetic PolyA over 45 base pairs in length, as well as synthetic DNA and polyglutamic acid – but not heparin sulfate – also led to increased PrP^{Sc} as assessed by Western blot, suggesting that a variety of polyanions can act as scaffolds for PrP^{C} conversion. These scaffolds could serve as an amplifier of PrP^{Sc} in vivo, although this remains to be shown, but these results may also be taken as a hint for amplifying prions in vitro which may be exploitable in diagnostic assays.

Increasingly, studies support oligomeric aggregates as the most toxic principle in amyloid diseases. New data from Byron Caughey's group (Hamilton, Mont., USA) incriminate a prion oligomer as the most infectious agent as well. Using fragmentation and fractionation, the most infectious particles/units with the highest converting activity were shown to be PrP oligomers, 300–620 kDa in size and corresponding to a 14–28 mer of PrP. This important clue provides a target size for those developing interference strategies to disrupt prion conversion.

Another hint for therapeutic design may be found in the interaction sites for PrP^{C} and PrP^{Sc}. These sites have been mapped (A. Williamson, San Diego, Calif., USA) by grafting PrP epitopes onto a human IgG antibody scaffold to determine epitopes that precipitate PrP^{Sc} and infectivity. The critical components of the interaction were determined to be between PrP amino acids 89–112 and 136–158. Similar strategies may provide a more detailed characterization of the PrP^{C}–PrP^{Sc} interaction site in the future.

Location Location Location: Where Does Cellular Conversion Take Place?

Cellular compartments for prion conversion recognized previously have included the plasma membrane and lysosomes, but now we have evidence from D. Harris' group (Washington University, St. Louis, Mo., USA) that the Golgi apparatus may be involved (presented by S. Barmada). A transgenic (TG) mouse with a GFP tag at the C-terminus of PrP has been generated to investigate PrP^{Sc} localization and trafficking. Although the PrP-EGFP was not converted to PrP^{Sc}-EGFP, it nevertheless did interact with PrP^{Sc} intracellularly, and the resulting PrP-EGFP:PrP^{Sc} complex accumulated in the Golgi. Perhaps the Golgi is another site where PrP converts. Serial transmission of these PrP-EGFP:PrP^{Sc} complexes into new PrP-EGFP mice, may result in adaptation and potentially break of the apparent 'species-barrier', leading to the desired PrP^{Sc}-EGFP.

In vitro studies have shown that scrapie infection of cells has an acute phase, where prion infection occurs independently of cell type or TSE strain, which is followed by a chronic phase where the cell type and TSE strain are

crucial for inducing a persistent infection (S. Priola, Hamilton, Mont., USA). Indeed, the PrP^{Sc} Western blot banding pattern changes over time in the prion-infected cell lines. Additionally, the subcellular compartment for PrP^{Sc} amplification may differ among cell type. The replication site as well as other yet unknown cellular factors may determine whether a particular cell type supports a chronic infection.

Neurotoxicity: The Usual Suspects

Are we any closer to understanding the mechanism of prion neurotoxicity? Cytosolic PrP, proposed as misfolded PrP retrotranslocated into the cytoplasm from the endoplasmic reticulum, has been suspected to be a neurotoxic intermediate form of PrP [5, 6]. In vivo evidence of this toxicity was presented using a TG mouse model with tetracycline inducible expression of a cytosolic PrP (Jiyan Ma, Columbus, Ohio, USA). The TG mice developed insoluble aggregates from the transgene, cytosolic PrP, which could be precipitated, while the endogenous cellular PrP was shown to be soluble. Indeed, the cytosolic PrP was toxic to neurons, and the TG mice developed neurologic disease. An important mouse line (work in progress) expressing a cytosolic version of another GPI-linked brain protein might prove or disprove the specificity of the described PrP toxicity.

Another molecular intermediate with neurotoxic properties is a transmembrane form of PrP, ^{Ctm}PrP, which is generated in small amounts and can span the lipid bilayer. A TG mouse model of transmembrane prions has been produced by David Harris' group (Washington University, St. Louis, Mo., USA) and, like the mouse with cytosolic PrP, also develops neurologic disease (presented by R.S. Stewart). Mice that also express wild-type PrP demonstrate a loss of cerebellar granular cells, but no misfolded PrP^{Sc}. Surprisingly, TG mice on a PrP–/– background were protected – mice no longer developed neurologic disease or any signs of CNS pathology. In addition, there was a dose effect seen with co-expression of endogenous, wild-type PrP; the higher the PrP expression, the earlier the neurologic phenotype was observed. This finding suggested that the ^{Ctm}PrP interferes with the function of endogenous PrP. Is transmembrane or cytosolic PrP a component of neurotoxicity in natural TSEs? Perhaps the neurotoxic species of PrP will be further unraveled in future TSE meetings.

In vivo Studies Provide Mechanistic Insights

The use of TG mouse models has provided a key tool to complement in vitro studies of prion disease investigation. In recent years, in vitro studies have revealed that although the majority of PrP is expressed on the cell surface via a GPI linker, a smaller amount of anchorless PrP is produced and secreted. To address whether the anchorless PrP accelerates or inhibits conversion, a TG mouse model that expresses solely GPI-anchorless PrP has been generated (B. Chesebro, Hamilton, Mont., USA). Perhaps surprisingly, exposure of these mice to scrapie prions leads to the development of PrP plaques in the brain, but without neurologic disease. However, mice that express wild-type PrP in addition to the anchorless PrP develop scrapie with an accelerated course of disease, showing that anchorless PrP contributes to disease.

The question of whether PrP glycosylation influences prion disease was addressed using knock-in mice. PrP has two glycosylation sites whose variable occupation results in 3 PrP subtypes: unglycosylated, monoglycosylated, or diglycosylated. TG mice with absence of either a single glycosylation site or both glycosyl moieties were all susceptible to TSE infection although not to the same extent. The glycosylation of host PrP affected the incubation period of disease and targeting of pathology within the CNS. (J. Manson, Edinburgh, UK).

Prion disease in mice, similar to variant CJD or chronic wasting disease, typically has an early lymphoid phase where prions accumulate in germinal centers, prior to invasion of the CNS. A recent discovery using several mouse models has revealed that prions can also replicate in inflammatory foci in nonlymphoid organs, such as liver, pancreas, or kidney, provided the inflammation consists of tertiary follicles and follicular dendritic cells (A. Aguzzi, Zürich, Switzerland). Tertiary follicles generated via lymphotoxin signaling supported prion replication, whereas nonfollicular inflammation in lymphotoxin knock-out animals was devoid of prion replication outside of secondary lymphoid organs. Indeed, lymphoid follicular inflammation (via LT signals), defined the sites where prions replicate, and has potential consequences for food safety, and implications for prion secretion from inflamed organs [7].

In another aspect of prion pathogenesis, the role of DCs in prion transport was addressed using skin scarification and topical prion exposure (N. Mabbott, Edinburgh, UK). In 2 models, Langerhans dendritic cell (LC) migration was inhibited using either a CD40L–/– mouse or through caspase 1 inhibition. Scrapie infectivity

reached the draining lymph node regardless of whether LC migration was inhibited or not, suggesting that LCs are not major prion transporters.

Genetic Features of Prion Diseases

To address questions of prion strains and species barriers, several PrP knock-in TG mouse models have been generated (J. Manson). Mice with a leucine at PrP codon 101, mimicking a familial mutation causing Gerstmann-Sträussler-Scheinker syndrome in humans, did not develop a spontaneous prion disease as seen in humans, but the mutation did lead to a decrease in incubation times across three species barriers when mice were inoculated with hamster 263K, sheep SSBP/1, human Gerstmann-Sträussler-Scheinker syndrome.

Notes from the Field

An unusual case of CJD has been reported by Gianluigi Zanusso (Verona, Italy). The patient had no PrP gene mutation, PrP genotype at codon 129 (polymorphic site) was 129MV heterozygous, MRI was negative for typical CJD lesions, and the brain pathology was similar to PrP genotype 129VV, with large amounts of intracellular PrP. The PrP^{Sc} Western blot pattern was highly unusual in that there was no diglycosylated band. Additionally in Italy, an atypical BSE case has been described and designated *bovine amyloidogenic spongiform encephalopathy* or BASE [8]. This case potentially represents a novel strain of bovine prion disease. It has a Western blot glycoform pattern with a predominant monoglycosylated band (in contrast to diglycosylated band in BSE), an unusual pattern of PrP^{Sc} accumulation in the brain (for example, abundant deposition in the olfactory bulb versus brainstem in typical BSE) and an older age distribution in the cattle. Mouse inoculation studies are in progress to complete the strain characterization.

Improving Our Diagnostics: Progress

Recent studies in humans with sporadic CJD have revealed PrP^{Sc} in the muscle [9], presenting a relatively noninvasive procedure for diagnosing human TSEs without a brain biopsy (M. Glatzel, Zürich, Switzerland). Additional work in diagnostics has included attempts to derive a prion disease biomarker from large-scale transcriptomic analyses of gene expression at different stages of mouse scrapie infection (G. Miele, Zürich, Switzerland). Thus far, the most profound increase in CNS tissue mRNA (over $50\times$) has been associated with the gene for cystatin F.

Treatment of Prion Diseases, Reason for Hope

Although past studies have conveyed hope for a possible anti-PrP vaccination and/or antibody therapy, more recent data illustrating a strong immunological tolerance to PrP [10], as well as the potential for unwanted immune-mediated neurotoxic effects, have presented substantial barriers to these strategies. Indeed, direct brain injection of anti-PrP antibodies targeting prion epitope 90–105 leads to PrP cross-linking and neuronal loss (A. Williamson). Therefore, although antibodies may ultimately prove useful for the prevention or treatment of prion disease, considerable hurdles have arisen which have dampened early enthusiasm [11].

On a more positive note, surprising results were shown using TG mice in which PrP^{C} expression is ablated at ~12 weeks of age by endogenous transgene activation (G. Mallucci, London, UK). When PrP in neurons is depleted in mice with established CNS scrapie infection, there is reversal of early spongiform degeneration and the mice survive long term, despite abundant extraneuronal PrP^{Sc} deposition [12]. This work suggests that PrP depletion strategies would be worthwhile to investigate for prion disease therapies.

Additional hope for treatments stems from studies with an inhibitory molecule, PrP-Fc_2, consisting of a human immunoglobin Fc fused to 2 PrP^{C} molecules (A. Aguzzi). Previous published work showed that PrP-Fc_2 prevents prion replication in TG mice expressing PrP-Fc_2 and wild-type PrP [13]. Follow up studies in wild-type mice have revealed that PrP-Fc_2 administered in a lentiviral vector at the time of prion inoculation delays prion replication and disease. The PrP-Fc_2 lentiviral delivery is planned to be submitted for a small-scale phase 1 clinical trial.

The multidisciplinary nature of prion science creates a charged atmosphere for a meeting, with lively discussions about the basic questions still persisting in the field, including the function of the PrP, the mechanism of neurodegeneration and the nature of prion strains. Meetings like the one reported here serve as chaperones that bring together investigators from various scientific disciplines, whose interaction may lead to generation of new ideas for experiments that will shed light on the fascinating issues of prion biology and disease.

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